SUPEROXIDE DISMUTASE IN LEUKOCYTES

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1. Introduction

Superoxide dismutase (SOD), an enzyme that catalyzes the dismutation of superoxide anion radical (O_2^-) to hydrogen peroxide and oxygen [1], has been isolated from several organisms, both procaryotes [2,3] and multicellular [1,4,5] or unicellular [6] eucaryotes. SOD has been implicated as being an essential defense against the toxicity of superoxide anion radical [7]. It has also proved to be useful as a probe for the involvement of O_2^- in a variety of reactions [8–12].

Using superoxide dismutase Babior et al. have demonstrated the production of O_2^- in phagocytosing human leukocytes [13]. Therefore, in view of the occurrence of SOD in a wide range of living organisms and of its postulated role as a defence against O_2^-, one would expect that also leukocytes contain this enzyme.

Assays of SOD activity are indirect and depend upon its ability to scavenge O_2^- from reaction mixtures and thus to inhibit reactions caused by O_2^-, for instance reduction of cytochrome *c* or Nitro Blue Tetrazolium (NBT) by O_2^- generated in the xanthine oxidase reaction [1,14]. Using both these assays we have shown the occurrence of SOD activity in guinea pig leukocytes.

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2. Materials and methods

Leukocytes were obtained from sterile guinea pig peritoneal exudates as described previously [15]. Leukocyte suspensions were always subjected to hypotonic treatment with 0.2% NaCl, in order to hemolyze contaminating red cells, before homogenization. Cells (4×10^8/ml) were homogenized in 1 mM phosphate buffer pH 7.8 in a Potter type homogenizer with a Teflon pestle driven by a motor. The homogenate was centrifuged at 100 000 *g* for 20 min and the supernatant fluid, after suitable dilution, was used to assay SOD activity. This was measured by following the inhibition of reduction of cytochrome *c* [1] or NBT [14] in the xanthine–xanthine oxidase reaction. The reduction of NBT and cytochrome *c* was followed at 560 and 550 nm respectively. One unit of SOD activity is defined as the amount of enzyme that causes a decrease of the optical density from 0.025 to 0.0125 in 1 min using 1 cm light path cuvette.

Disc gel elect ophoresis was performed according to the method of Davis [16]. The protein bands were stained using Coomassie blue. Superoxide dismutase activity was detected on the gels according to the method of Beauchamp and Fridovich [14]. Catalase activity was measured as oxygen liberated from H_2O_2, produced by glucose–glucose oxidase reaction, upon addition of the catalase-containing sample [17]. Hemoglobin was assayed by the cyanmethemoglobin method [18].

Cytochrome *c*, NBT and glucose oxidase were obtained from Sigma. Xanthine oxidase was obtained from Boehringer.

3. Results

Fig. 1 and 2 shows the inhibition of the reduction of cytochrome *c* and NBT respectively but the 100 000 *g* supernatant fluid of PMN homogenates. Inhibition was abolished if boiled supernatant was used suggesting the protein nature of the substance responsible for the inhibition. Table 1 shows the distribution of superoxide dismutase and catalase activities among the ammonium sulfate fractions obtained from the 100 000 *g* supernatant fluid of PMN homogenates. This supernatant contained no more than 0.1% of the total myeloperoxidase (MPO) activity of the original homogenate. The bulk of MPO activity remained bound to granules after homogenization. This ruled out the possibility that O_2^- may be scavenged by MPO to form compound III [19]. Most of the superoxide dismutase activity was found in the 90% ammonium sulfate fraction whereas catalase was predominantly found in the 60% ammonium sulfate fraction.

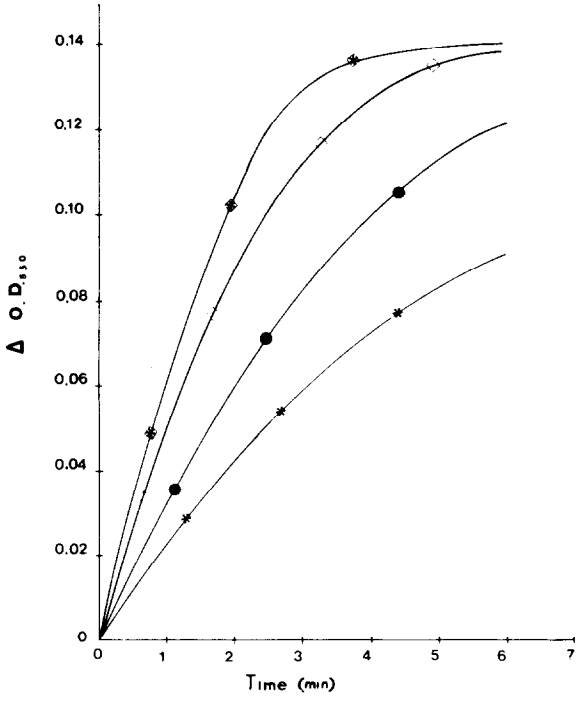

Fig. 1. Inhibition of the reduction of cytochrome *c* by xanthine–xanthine oxidase reaction in the presence of the 100 000 *g* supernatant of guinea pig leukocyte homogenate. The assay mixture contained 50 μM xanthine, 10 μM cytochrome *c*, 0.01 Unit xanthine oxidase, 0.05 M phosphate buffer pH 7.8 and 0.1 mM EDTA. Final volume 3 ml. Temperature 25°C. ▣——▣ Control. *——* Supernatant from 6 X 10^7 cells. ●——● Supernatant from 3 X 10^7 cells. □——□ Boiled supernatant from 6 X 10^7 cells.

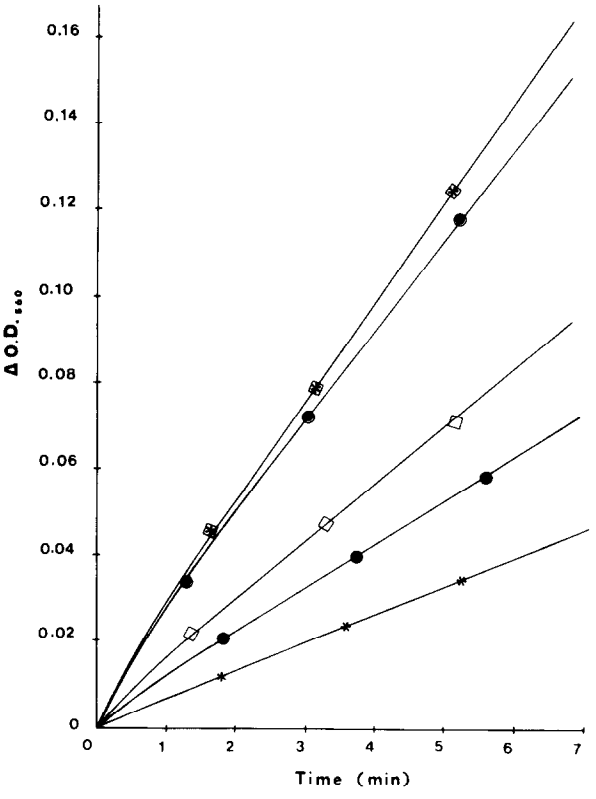

Fig. 2. Inhibition of the reduction of Nitro Blue Tetrazolium (NBT) by xanthine-xanthine oxidase reaction in presence of the 100 000 *g* supernatant of guinea pig leukocyte homogenate. Assay as in fig. 1 except that cytochrome *c* was replaced by 50 μM NBT. ▣——▣ Control. *——* Supernatant from 6 X 10^7 cells. ●——● Supernatant from 3 X 10^7 cells. □——□ Supernatant from 1.5 X 10^7 cells. ⊙——⊙ Boiled supernatant from 6 X 10^7 cells.

3.1. *Detection of SOD on acrylamide gels*

Aliquots of the 90% ammonium sulfate fraction were subjected to polyacrylamide disc gel electrophoresis and the gels stained for SOD activity, using the photochemical reduction of NBT according to Beauchamp and Fridovich [14], or for protein. In the staining for enzymatic activity the gel becomes uniformly blue except in the site of SOD where an achromatic zone is visible. The results of the densito-

Table 1
Ammonium sulfate fractionation of the 100 000 *g* supernatant of guinea pig leukocytes. Distribution of superoxide dismutase (SOD) and catalase activities

	SOD Units/ 6×10^7 cells	Catalase n atoms 0 liberated/min/ 6×10^7 cells
Whole supernatant	2.510	74.6
Ammonium sulfate fraction 1	0.152	4.8
Ammonium sulfate fraction 2	0.232	68.6
Ammonium sulfate fraction 3	2.192	0.0

SOD was assayed using cytochrome *c* as an acceptor as described in fig. 1. Catalase activity was assayed by monitoring with a Clark oxygen electrode the oxygen liberated, after addition of the sample, from H_2O_2 enzymatically produced (2 mg glucose and 0.4 units glucose oxidase in 2 ml of 0.05 M phosphate buffer pH 7.8. Temperature 37°C). Fractions 1,2 and 3 were the precipitates from 0–80%, 30–60%, 60–90% ammonium sulfate saturation respectively. The ammonium sulfate fractions were precipitated at 100 000 *g* per 15 min.

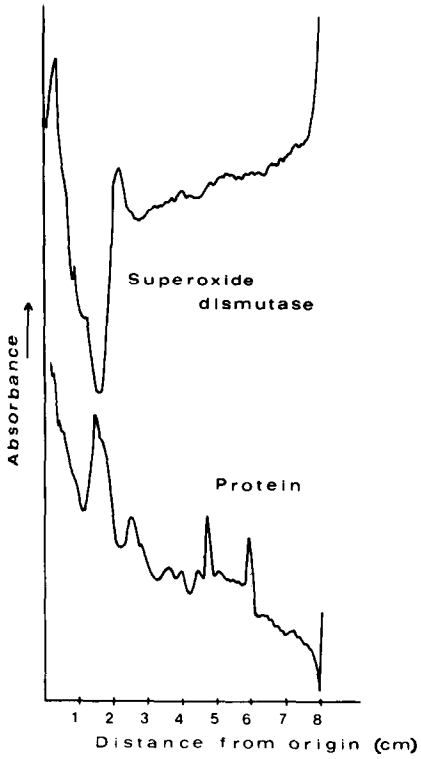

Fig. 3. Densitometric scanning profiles of the polyacrylamide gel electrophoretograms of the 90% ammonium sulfate fraction of the 100 000 *g* supernatant of guinea pig leukocyte homogenate after staining for SOD (top) and protein (bottom). About 50 μg protein were applied on the gels. The gels were 5 mm in diameter and 6 cm long, 5% cross linked and were run at 2 mamp/gel. The run was stopped when the marker Bromophenol Blue had swept through most of the gel. For the protein stain the gels were soaked in Coomassie Brilliant Blue (0.25% Coomassie Brilliant Blue, 9% acetic acid, 45% methanol) for 36 hr and then destained in 7.7% acid containing 5% methanol. For the SOD stain the gels were soaked in 2.45×10^{-3} M NBT for 20 min and then in a solution containing 0.028 M tetramethylethylenediamine, 2.8×10^{-5} M riboflavin and 0.036 M potassium phosphate pH 7.8. The gels were then placed in a dry test tube and illuminated. Illumination was discontinued when the contrast between the achromatic zone and the general blue color was maximum. During scanning chart was run at 5 cm/min and gel at 4 cm/min.

metric scanning of the gels stained for SOD or protein are shown in fig. 3. SOD is localized in a slow-moving band that corresponds to the achromatic zone on gels stained for enzymatic activity.

3.2. *Contamination by the erythrocyte SOD*

Guinea pig erythrocytes have a SOD activity of 40.0 units per 3×10^8 cells or 6.6 units per 1.0 absorbance of cyanmethemoglobin at 540 nm (our measurements; cytochrome *c* used as electron acceptor). Treatment of erythrocytes with a 0.2% solution of NaCl for 2 min released virtually 100% of both hemoglobin and SOD. Before homogenization our PMN suspensions were pretreated with 0.2% NaCl for 2 min in order to remove SOD of contaminating erythrocytes. The mean SOD activity of our preparations was 10.63 ± 0.46 SEM (7 experiments) per ml of supernatant (3×10^8 cells/ml). If this activity were contributed by the erythrocyte enzyme, the cyanmethemoglobin reading at 540 nm should have been 1.6 absorbance. In our experiments the highest cyanmethemoglobin reading obtained was 0.020 absorbance per ml of supernatant indicating that maximal contamination by the erythrocyte enzyme would be about 1%.

4. Discussion

The role of SOD in leukocytes seems to be more complex than it may be in other cells. In fact O_2^- in leukocytes may not only exert a harmful effect, as it

is in other cells, due to its ability to initiate free radical chain reaction, but may also play a protective role due to its bactericidal activity. Indeed a bactericidal activity of O_2^- has been postulated by Allen et al. [20] and by Babior et al. [13], and has been demonstrated directly by Lavelle et al. in vitro with *E. coli* [21].

Therefore dismutation of O_2^- by SOD in leukocytes would not only protect cell structures from O_2^--induced damage, but, in principle, could also reduce or control the bactericidal activity of the cell. It should be recalled, however, that O_2^- is not the sole bactericidal agent in leukocytes and that H_2O_2, the product of O_2^- dismutation, it itself bactericidal in conjunction with myeloperoxidase and halides [22–23].

One facet of the general protective role of SOD may be in leukocytes that of preventing free-radical damage to the granule membrane and hence the release of granule content and other cytoplasmic factors from the cell. Granule hydrolases are regarded as the effectors of tissue injury at the site of the inflammatory process [24], and other factors released from leukocyte granules are involved in such biological functions as chemotaxis [25], activation of the complement system [26], activation of the kinin system [27], and lymphocyte transformation [28] that are hallmarks of the inflammation.

A lysosomal damage caused by a free radical-like component, produced during NADPH oxidation by microsomes, has been shown to occur in liver by Chen and McCay [29]. Oxidation of NADPH by phagocytosing leukocytes is one of the main biochemical events that accompany phagocytosis [30–32] and may well be the source of O_2^-. The effect of O_2^-, produced in leukocytes by this or other mechanisms, on the stability of granule membrane and the putative protective role of endogenous SOD are under investigation. Should such a role receive experimental support the leukocyte O_2^--SOD system might be regarded as a modulator of those functions of leukocytes that are related to the evolution of the inflammatory process.

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